Identification of water stress responsive genes in coconut (*Cocos nucifera* L.)

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1. INTRODUTION

Drought and water stress are important abiotic stress factors having high negative impact on the yield of crop plants. Various factors at the level of morphological, anatomical, physiological and biochemical level in coconut have been attributed for the water stress tolerance (Rajagopal*et al*., 1989, 1996; Naresh Kumar *et al.*, 2000; Naresh Kumar *et al*., 2003). The biochemical response of coconut palm to drought stress include up-regulation or synthesis of scavenging enzymes to maintain cell membrane integrity thus enabling cells to tolerate stress. It is clear that the drought tolerant varieties are endowed with a biochemical mechanism to adapt the adverse effect of drought by appropriate regulation of enzyme activities. In coconut, lipid peroxidation was high in drought susceptible cultivars as compared to tolerant ones (Chempakam*et al*., 1993). Drought tolerance is characterized by higher activities of the protective enzymes like SOD, catalase and peroxidase and lower activity of phosphatase and consequently coupled with lower levels of lipid peroxidation and higher membrane integrity (Shivashankar*et al*., 1991; KasturiBai*et al*., 2003). Studies on genotyping and correlating with drought tolerance have been done with reciprocal cross progenies (Manimekalai*et al*., 2004).

Plants are uniquely suited for coping with periods of severe water deficit during certain stages of their life cycle. During the late phase of embryo maturation, embryos undergo severe desiccation resulting in a dry, mature embryo that can survive in a quiescent phase for longer periods. However, exposure of plants to water-limiting environments during the plant's vegetative, reproductive, or early embryo development phases appears to trigger a set of physiological and developmental changes. These are characterized by a number of biochemical changes that ultimately result from a selective increase or decrease in the biosynthesis of a large number of distinct proteins that alter enzyme activity. Changes in the protein profile are due to changes such as transcription rate, RNA stability, posttranscriptional control, and protein turnover (Smirhoff and Colombe, 1989). Several genes have been described that respond to dehydration at the transcriptional level in a variety of plant species (Tabaeizadeh, 1998). Although several genes induced by drought have been identified in a wide range of plant species (Bray, 1997; Tabaeizadeh, 1998), a molecular basis for plant tolerance to water stress remains unclear (Ingram and Bartels, 1996). More than 65 plant dehydrin genes have been sequenced (Close, 1997).

Even though extensive research led to delineation of drought tolerance mechanism in coconut (Rajagopal and KasturiBai, 2002; NareshKumar *et al.*, 2000; Rajagopal and Naresh Kumar, 2003), little is known about molecular events regulating gene expression under drought conditions. It is important to analyze drought-responsive gene expression in water-stressed and irrigated coconuts, as it may increase our understanding of the molecular mechanism of water stress and the role of differential gene expression in drought tolerance.

Detailed studies have been carried out on genes expressed during drought in crops like rice, barley, sunflower etc. Gene expression pattern in relation to water stress have been conducted in number of other crops *viz*. rice, barley, sunflower and citrus (Sanchez *et al.*, 2004; Ouvrard*et al.,* 1996). Numbers of genes that respond to abiotic stress at the transcriptional level have recently been described (Amudha and Balasubramani, 2011; Hussain*et al*., 2011). In barley, ABA induced protein kinase (PKAB1) was reported (Gomez-Cadenas*et al*., 1999) which may function in signal transduction pathway. In citrus, gene dehydrin gene expression was detected for the stress tolerance (Sanchez *et al*., 2004). Quin*et al*., (2004) cloned and analyzed a novel DREB1/CBF transcription factor involved in cold responsive gene expression in *Zea mays* L. Denekamp and Smeekens (2003) have shown the involvement of wounding and osmotic stress in the expression of AtMYB 102 transcription factor.

But no such work has been carried out in perennial crops like coconut, even though the HSP expression has been studied (Naresh Kumar *et al.,* 2007). Genes that are expressed during stress conditions will provide knowledge about the molecular aspects of response of coconut to the stress. Molecular markers based on gene expression pattern are vital for the perennial crop like coconut. In coconut, drought screening is based on morphological, anatomical, physiological and biochemical parameters. But if we have the molecular markers developed from the expressed genes, that will be used in development of Expressed Sequence Tag (EST) markers and hybridization based functional molecular markers for stress tolerance.

The aim of the present study is to identify genes which are expressed under drought stress in coconut. Specific transcripts uniquely affected due to water stress can be used as markers for selecting drought tolerant lines. The expression of these down-regulated transcripts in a drought tolerant and a susceptible genotype will be compared to identify putative transcripts that can be used as molecular markers in screening coconut genotypes for drought tolerance.

Hence the present study was started with following objectives:

1. To study the physiological and biochemical responses of seedlings exposed to water stress.
2. To design primers for water stress responsive genes based on conserved regions of stress responsive genes available in public sequence databases.
3. To amplify candidate stress responsive genes in coconut using the designed primers and to carry out homology studies of the derived sequences.
4. To study the gene expression during induced water stress using differential display analysis.
5. To design functional markers in coconut by sequencing the differentially expressed genes.

2. METHODOLOGY

Water stress is one of the most critical environmental stresses reducing coconut yield in rain-fed areas. It is therefore imperative to understand the molecular basis of genes imparting tolerance to water stress which will help in development of functional markers for identification of coconut cultivars with lower water requirements. In order to meet the objectives, two sets of experiments were conducted. In the first one, zygotic embryo cultured coconut plantlets were used and in the second experiment coconut seedlings were used. Broadly, two approaches were used for the development of functional markers for water stress responsive genes in coconut. In the first approach differential display technique was used while in the second one *in silico* probe designing and *in vitro* amplification of candidate genes responsive to water stress was carried out. Investigation of physiological and biochemical changes that occur during water stressed condition in coconut seedlings was also studied.

2.1. Coconut embryo culture: The *in vitro* culture of 350 embryos of WCTcultivarwas initiated in June, 2007. Surface sterilization of the embryos extracted from split open nuts was done with 20% sodium hypochlorite solution and with sterile distilled water till the traces of chlorine were removed. The Y3 medium (Eeuwens, 1976) supplemented with 30 g/L of sucrose formed basal medium and standardized protocol available for the coconut embryo culture (Karun *et al*., 1999) was followed.

2.2. Induction of water stress on plantlets: Six month after growth, half of plantlets were subjected to water stress induced by polyethylene glycol (6000 MW). Twohigher concentrations of filter sterilized PEG (20% and 30%) was applied to sterilized full strength Eeuwens Y3 liquid medium. Subsequently two plantlets each were sub-cultured into PEG containing medium. Apart from this, one pair of plantlets was also inoculated into Y3 medium without PEG, which served as the control plantlets.

2.3. Standardization of RNA isolation protocol: For standardization of RNA isolation, tissues of leaves from two year aged seedlings, leaves and root from embryo cultured plantlets were used. The RNA mini kit (Invitrogen), Trizol reagent (Sigma Aldrich, USA) and RNeasy plant mini kit (Qiagen, Germany) were used to find the best combination for RNA isolation.

2.4. mRNA differential display analysis: The mRNA differential display analysis was carried out according to Liang and Pardee (1992) method with slight modifications (Kavitha and George, 2008). Differential display polymerized chain reaction was performed on the reverse transcribed products using the corresponding oligo (dT) primers and random primers. The differential display analysis was carried out using three anchor primers $HT_{11}G$, $HT_{11}C$ and $HT_{11}A$ which were used in first strand synthesis and 70 combinations of the anchor primers were made with seven 13-mer arbitrary primers (HAP01-HAP07) from the RNAimage kit (GenHunter) and a total of 24 random decamer primers (OPB1-5, 9-10, 13-16, 18 and OPF 1-12) from Operon kit B and kit OperonF (Operon technologies, Alameda, CA).The PCR products were fractionated on a 6% polyacrylamide gel which was autoradiographed using Phosphor Imager (Bio-Rad) and then X-ray film was developed and the autoradiogram was aligned to the gel and the differential bands were eluted. Re-amplification PCR was performed on the differentially expressed fragments which eluted with the corresponding oligo (dT) primers and random primers.

2.5. Water stress treatment on seedlings: Thegerminated seed-nuts of WCTwere transplanted onto cement pots and after establishment of seedlings (~one year age seedlings) water stress treatments was imposed. For one set of 20 seedlings, irrigation was withdrawn, while another set of 20 seedlings were maintained under water non-limiting conditions to serve as control. Physiological and biochemical readings were taken at the beginning of

imposition of water stress and subsequently at 15 and 30 days after withdrawal of irrigation. DNA and RNA were isolated after 30 days water stress induction.

2.6. Observations on physiological parameters: Gas exchange parameters such as net photosynthetic rate, stomatal conductance, transpiration rate, leaf internal carbon dioxide concentration, water use efficiency were recorded using IRGA (LI-Cor 6400, USA). Leaf water potential was estimated using pressure chamber (SKPM 1400, UK) and soil moisture content was estimated gravimetrically.

2.7. Estimations of biochemical parameters: The total proteins as per Bradford's (1976) method and heat stable protein fraction as standardized for coconut leaf tissue (Naresh Kumar *et al*., 2007) were estimated. Activities of scavenging enzymes like Superoxide dismutase (Beauchamp and Fridovich, 1971), Peroxidase and Polyphenol oxidase (Kar and Mishra, 1976) and free proline (Bates *et al.*, 1973) werequantified

2.8. Extractionof DNA: Total genomic DNA was extracted from both water stressed and control WCT seedlings after a 30 days water stress induction using the modified SDS method for coconut leaf tissues (Upadhyay*et al*., 1999) and each purified genomic DNA was diluted to 10 ng/µL and stored at 4°C.

2.9. Primer designing: The candidate genes which are activated during water stress reported in plant species was collected via literature survey and the information was used for the *in silico* probe design and *in vitro* gene amplification. Based on these, selected genes were *AP2* (Apetala2),*Aquaporin*, *CBF* (C repeat binding factor), *CER2* (Eceriferum 2), *DREB (*Dehydration Responsive Element Binding*)*, *GL15* (Glossy 15), *KNOX* (Knotted-1 like homeobox), *MADS Box*, *MAPK* (Mitogen Activated Protein Kinase), *NAC* (NAM**,** ATAF, and CUC), *SERK* (Somatic Embryogenesis Receptor Kinase), *Wax2* (cuticular wax gene), *WRKY* (WRKY transcription factor) and *14-3-3* (14-3-3 transcription factor). Apart from these, two well-known reference housekeeping genes viz., *actin* and *rbcL* (Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) were chosen reference gene amplification. The nucleotide sequences of selected 16 genes reported in other plant species were retrieved from nucleotide sequence database GenBank, found at NCBI. Multiple sequence alignment was performed to the selected gene sequences with ClustalW. A set of primers were designed for the desired gene from the conserved regions of these sequences

using FastPCR software. The quality and specificity of the designed primers were validated with using both PrimerBLAST and FastPCR analysis.

2.10. Primer screening: The *in vitro* amplification of synthesized oligos in coconut genomic DNA performed with DNA isolated from leaf tissue of West Coast Tall seedlings. The optimum annealing temperature for each primer pair was worked out by using 12 gradient annealing temperatures ranging from 45°C to 65°C. Amplicons with expected band size was again amplified in conventional PCR with best annealing temperature obtained for the primer pair. The eluted, cleaned amplicons were sent for DNA sequencing along with corresponding primer set. Homology search was performed for the sequenced samples using BLAST algorithm.

2.11. Polymerized chain reaction: The DNA was isolated from coconut seedlings 30 days after withdrawal of irrigation and also from control seedlings. These DNA samples wereused for conventional PCR amplification.

2.12. RNA isolation: Total RNA was isolated from both control and water stressed (30 days after withdrawal of irrigation) WCT seedlings via RNeasy plant mini kit (Qiagen, Germany). The concentration of total RNA isolated from each sample was determined spectrophotometrically at 260 nm (Bio-Rad, USA).

2.13. RT-PCR: The primer pairs which are amplified and got homology in conventional PCR amplifications were used in water stressed gene expression studies in coconut seedlings. A total of 10 primer pairs representing 10 candidate genes for water stress were used in RT-PCR amplification. Two housekeeping genes actin and rbcL were also tried as reference gene amplification.

2.14. Agarose gel electrophoresis: Agarose gel electrophoresis was used to separate, identify, and purify DNA fragments. After conventional PCR and RT-PCR (reverse transcriptase) amplification, the products were resolved in 1.2% agarose gel using 1X TBE buffer. Agarose gel (0.8%) was used for total genomic DNA check and amplicons excise 1.5% agarose gel was used for total RNA check.

2.15. Amplicons elution: For amplicons elution, the amplified PCR products were separated on 0.8% agarose gel and clear high intensity amplicons were excised under UV-transiluminator (Bio-Rad). The excised amplicons were purified by usingQIAquick® gel extraction kit (Qiagen, Germany) and GenElute gel extraction kit (Sigma, USA).

2.16. Cloning: Cloning of the eluted fragments was carried out using the InsTA clone™ PCR product cloning kit (Fermentas). The desired amplicons were ligated into pTZ57R/T vector using T4 DNA ligase and incubated the ligation mixture at 37°C for six hours. The ligated PCR product containing vector was transformed into competent cells of *E. coli* strain DH5α prepared by ice chilled treatment. After ligation and transformation, the cells were finally plated on pre-warmed LB-agar plates containing 50 µg/mL of ampicillin, 100 ppm of IPTG and 160 ppm of X-gal and thereafter plates were incubated overnight at 37ºC. The recombinant white clones were selected and subculture into new LB-agar plates containing same concentrations of ampicillin, IPTG and X-gal. The pure white clones identified in LB-agar plates were transferred into ampicillin containing LB-broth. Colony PCR was performed for the conformation of presence of inserted genes before samples sent for DNA sequencing. Finally, colony PCR confirmed positive clones were sent to outsource for DNA sequencing and was sequenced at Chromous Pvt. Ltd., Bangalore.

2.17. Homology based gene prediction: The DNA sequences obtained by RT-PCR and DDRT-PCR amplifications were performed for a couple of genome annotations. In first step vector contaminated regions from the cloned sequences were removed with the help of VecScreen tool provided by NCBI. Sequence level similarity was estimated using the local alignment algorithm BLAST search programme. Pair-wise alignment was carried out using the web based ClustalW with default parameters. A phylogenetic analysis of closely related species was carried out with homologous sequences obtained via the BLAST analysis. A rooted neighbour-joining phylogenetic tree was generated using the MEGA software package version 3.0.

2.17.1. EST-SSR analysis:A search for simple sequence repeat regions in expressed genes in water stressed condition was carried out. Cloned and sequenced amplicons derived through differential display were chosen for the SSR examination. A computational tool was developed in PHP programming language for the prediction of microsatellite regions (Paul *et al*., 2010).

2.18. Sequence submission: Genes, which isolated and characterized by RT-PCR were submitted to the nucleotide sequence database GenBank found in NCBI. Sequin was used to submit the sequences got maximum similarity and identity as per the instructions made by them. Sequences generated through DDRT-PCR analysis were submitted in dbEST, an EST database found at NCBI. Batch submission procedure was followed in case of DDRT-PCR sequences submission. Sequences were arranged in dbEST format and sent to dbEST database administrator.

2.19. Development of functional markers: Homology and E-value were the selection criteria for the devolvement of functional markers. The annotated and high homologues sequences showing distinguishable difference in control and water stress treated seedlings and found in SSR and EST were selected for functional marker development. A pair of gene specific primers was designed for selected gene sequence with the help of FastPCR software.

2.20. Web designing: Molecular data obtained from present study were put in an in-house data warehouse. A user friendly front end was designed with PHP coupled with HTML and back end was furnished with MySQL. Separate pages were created for reagents, molecular calculator and primer analyzer.

3. SUMMARY OF RESULTS

3.1. Differential display analysis: Analysis using the 70 primer combinations revealed 129 up-regulated transcripts to be differentially expressed. The transcript derived fragments (TDFs) were eluted, cloned and sequenced. Low-quality sequence regions were trimmed and sequences less than 75 bp in length were excluded. A total of 114 cloned, sequenced and differentially expressed fragments passed sequence screening and BLAST search was performed. Results revealed that several transcripts were significantly homologous to stress regulated genes/proteins isolated from other plant species. Highly similar sequence representing genes were selected and made into 10 groups based on their function and sub-cellular location. The hierarchy of grouped transcripts is as follows: enzymes from metabolic pathways (27.2%), regulatory genes (23.6%), protein family (15.7%), DNA binding domains (5.3%), hypothetical proteins (5.3%), contings (5.3%), chromosomes (5.3%), no similarity (5.3%), clones (4.4%) and transcription factors (2.6%). The results indicated induction of candidate genes during water stress in coconut. The fully analyzed EST sequences were deposited in dbEST database.

3.2. Observation on physiological parameters: The average soil moisture content of 31.3% was maintained in all control seedlings throughout the experimental period. On the other hand, in stressed seedlings initial soil moisture content was 31.7% and after 15 days of stress induction, soil moisture content lowered to 12.4% and after 30 days of stress, soil moisture content further lowered to 8.9%. Response of seedlings to water stress was assessed by measuring the gas exchange parameters and also leaf water potential. While leaf water potentialremianed almost stable in the range of -5.26 bars to -5.9 bars in irrigated seedlings, seedlings subjected to water-stress had significantly lower water potential and it decreased progressively with the duration of water stress. The net photosynthetic rate in irigated seedlings ranged from from 4.86 µmol/m^2/sec to 10.52 µmol/m^2/sec during the experimental period, while seedlings subjected to water-stress had initially higher Pn rates and then decreased significantly to 1.30 µmol/m^2/sec. Similar trends were observed for stomatal conductance, transpiration rate and Ci. The ratio between net photosynthetic rate and leaf internal CO_2concentration (Pn/Ci), a indicator for mesophyll efficiency, increased in irigated seedlings while it decreased in water-stressed ones. The ratio between leaf internal CO_2 to ambient CO_2, an idicator of stomatal resistnace, was higher in well watered seedlings than in water stressed seedlings.

3.3. Estimation of biochemical parameters: Water stress affected the concentration of leaf total proteins, which decreased by ~12.1% and ~29.8% after 15 and 30 days, respectively, of exposure to water stress. On the other hand, percentage of heat stable protein fraction (HSPF) in total proteins increased by 39% and 90.4% after 15 and 30 days, respectively, of exposure to water stress. However, in well watered seedlings no significant change was noticed in percentage of total leaf proteins or that of heat stable protein fraction. The activity of scavenging enzymes such as superoxide dismutase increased by 34%, peroxidase increased by 60% and poly phenol oxidase by 89.2% after 15 days of exposure to water stress as compared to the activity rates of respective enzymes in well watered seedlings. This activity further increased again, superoxide dismutase increased by 41.6%, peroxidase increased by 105.5% and poly phenol oxidase increased by 137.1% after 30 days of exposure to water stress.

3.4. Primer designing and candidate water stress gene amplification: A total of 53 primer pairs were designed and synthesized according to conserved regions found in 14 candidate water stress responsive genes and two reference housekeeping genes selected. Reverse-

transcriptase PCR (RT-PCR) using the designed primers was performed to study the expression patterns of the selected eight candidate genes in leaves of two-year old coconut seedlings subjected to water stress. All the eight genes were found to be up-regulated suggesting that these genes may contribute to adaptation of coconut to water stress.

Actin gene got maximum identity with *Elaeisguineensis* (94%) in BLASTN database search and BLASTX search showed maximum identity with *Oryza sativa* (87%). *AP2* gene got maximum identity with *Elaeisguineensis* (95%) in BLASTN database search and BLASTX search showed maximum identity with *Elaeisguineensis* (71%). *Aquaporin* gene got maximum identity with *Elaeisguineensis* (95%) in BLASTN database search and BLASTX search showed maximum identity with *Elaeisguineensis*(97%). *CBF* gene got maximum identity with *Rhapidophyllumhystrix, Attaleabasslerianaand Elaeisguineensis* (95%) in BLASTN database search and BLASTX search showed maximum identity with *Elaeisguineensis*(93%). *MAPK* gene got maximum identity with *Nicotianabenthamiana, Lycopersiconesculentum*and *Triticumaestivum* (85%) in BLASTN database search and BLASTX search showed maximum identity with *Zea mays, Oryza sativa, Triticumaestivum, Rheum australe* and *Ricinuscommunis* (94%). *NAC* gene got maximum identity with *Elaeisguineensis* (95%) in BLASTN database search and BLASTX search showed maximum identity with *Elaeisguineensis* (80%). *rbcL* gene got maximum identity with *Scheeleabutyracea, Allagopteraarenaria, Parajubaeatorallyi, Orbignyabarbosiana* and *Syagrusglaucescens* (98%) in BLASTN database search and BLASTX search showed maximum identity with *Orbignyabarbosiana, Attaleabutyracea, Allagopteraarenaria, Parajubaeatorallyi* and *Syagrusglaucescens* (99%).

Two primer pairs of SERK gene amplified in coconut with two fragments size. Accession No. HQ840419 got maximum identity with *Areca catechu* (97%) in BLASTN database search and BLASTX search showed maximum identity with *Paeoniasuffruticosa* and *Solanumperuvianum* (94%). Accession No. FJ694854 got maximum identity with *Arabidopsis lyrata* (91%) in BLASTN database search and BLASTX search showed maximum identity with *Oryza sativa* (95%). *WRKY* gene got maximum identity with *Syagruscampylospatha, Butiayatay, Attaleapindobassu* and *Jubaeachilensis* (98%) in BLASTN database search and BLASTX search showed maximum identity with *Voanioalagerardii, Syagrusvermicularis, Butiacapitata* and *Attaleaspeciosa* (100%). *14-3-3* gene got maximum identity with *Elaeisguineensis* (92%) in BLASTN database search and BLASTX search showed maximum identity with *Elaeisguineensis* (92%).

3.5. EST-SSR (Expressed Sequence Tag-Simple Sequence Repeats) analysis: A total of 114 ESTsequences obtained through differential display analysis were analyzed with simple sequence repeats (SSR) search tool. Result of the SSR search gave 84 sequences having notable repeats regions. The clone CNDD117C having maximum number of 11 repeating units and 30 EST sequences do not have at least single repeat unit. The SRR search on coconut EST sequences showed that mono-, di-, tri- and tetra-nucleotide constituted 46.86%, 43.47%, 8.70% and 0.97% , respectively, of total EST analyzed.

3.6. Sequence submitted in dbEST: Genes expressed during PEG induced water stressed conditions were identified through differential display analysis and were submitted to International Nucleotide Sequence Database Collaboration for free access. The resulting 114 sequences were submitted in dbEST database. The Accession numbers of deposited sequences are listed below:

JG390667, JG390678, JG390682, JG390691, JG390693, JG390695, JG390704, JG390706, JG390709, JG390711, JG390712, JG390716, JG390720, JG390725, JG390726, JG390730, JG390740, JG390741, JG390747, JG390758, JG390759, JG390761, JG390762, JG390766, JG390769, JG390771, JG390773, JG390663, JG390668, JG390669, JG390674, JG390675, JG390679, JG390683, JG390684, JG390686, JG390690, JG390694, JG390697, JG390698, JG390715, JG390719, JG390722, JG390723, JG390728, JG390729, JG390731, JG390733, JG390735, JG390737, JG390738, JG390753, JG390755, JG390757, JG390760, JG390772, JG390775, JG390776, JG390685, JG390688, JG390692, JG390724, JG390710, JG390734, JG390664, JG390666, JG390768, JG390774, JG390748, JG390745, JG390746, JG390718, JG390714, JG390702, JG390676, JG390700, JG390705, JG390713, JG390744, JG390689, JG390707, JG390736, JG390739, JG390673, JG390687, JG390665, JG390680, JG390699, JG390708, JG390727, JG390742, JG390703, JG390721, JG390732, JG390743, JG390750, JG390770, JG390677, JG390681, JG390696, JG390701, JG390751, JG390752, JG390670, JG390671, JG390672, JG390717, JG390763, JG390749, JG390754, JG390756, JG390764, JG390765, JG390767

3.7. Sequence submitted in GenBank: Candidate water stress responsive transcripts identified and characterized in coconut obtained via RT-PCR amplification were submitted in GenBank, a nucleotide sequence database maintained by NCBI. Eight water stress induced genes and two housekeeping genes from coconut were amplified through RT-PCR. The unique accession number of submitted gene sequences from coconut is given below:

FJ200378, FJ694854, HQ840419, HQ840420, HQ840421, HQ840422, HQ840423, HQ840424, HQ840425, HQ832420

3.8. Functional markers: The genome information obtained from the present study paves the way for functional annotation of candidate genes triggered during water stress in coconut. Fiftyhighly significant gene sequences based on the similarity, identity and E-value were chosen for designing functional markers, which will be used for future studies to screen coconut germplasm collection for tolerance/susceptibility for water stress. From these, 40 markers were designed from DDRT-PCR analysis and 10 markers were RT-PCR amplified.

3.9. Web designing: A user friendly website named 'Coconut water stress gene database' was created for analyzing and for accessing data generated in the present study. Separate page link were created for each subtitle named database, tools, molecular calculators, recipes and stress genes.

4. SUMMARY AND CONCLUSION

Water stress is one of the most important stresses that limit coconut production. Several morphological, anatomical, physiological and biochemical characteristics are reported to impart drought tolerance in coconut. However, no studies are available indicating the genes expression during water stress conditions, a knowledge that can help in developing marker assisted selection of tolerant plants. It is therefore important to identify water stress responsive genes in coconut and investigate their role under water stress and irrigated conditions in order to decipher molecular mechanism of differential gene expression under water stress conditions. Hence the present study was conducted with objectives to study the water stress responsive genes and to design functional markers by sequencing the differentially expressed genes.

In order to meet the objectives, two sets of experiments were conducted. In the first approach, zygotic embryo cultured coconut plantlets were subjected to water stress through PEG and leaf tissue from these plant lets was used to identify differential gene expression. In the second approach, seedlings were subjected to water stress, which was quantified by estimating physiological and biochemical parameters. *In silico* probe designing and PCR amplification of candidate genes responsive to water stress were carried out in these

seedlings. Water stress in seedlings was quantified using various physiological and biochemical parameters. Water-stress decreased net photosynthesis rate, stomatal conductance, leaf internal CO_2, transpiration rate, ratio between net photosynthetic rate and leaf internal CO_2, and ratio between leaf internal CO_2 to ambient CO_2 and total proteins in the leaves as a consequence of reduced leaf water potential. On the other hand, water stress increased heat shock protein fraction, scavenging enzymes (superoxide dismutase, peroxidase and polyphenol oxidase) and proline with period of exposure to stress. Sequence comparison of transcripts induced during water-stress in coconut, identified through differential display in BLAST database revealed induction of several transcripts significantly homologues to stress regulated genes/proteins from other plant species. Homology search of candidate genes identified through *in silico* primer design and PCR amplification showed high similarity (more than 90%) with stress responsive genes identified in other plant species. These results pave the way for identification and functional annotation of candidate genes triggered during water stress in coconut. Fifty highly significant sequences were chosen for design of functional markers, which will be used in future studies for screening coconut germplasm for tolerance to water stress.

5. PUBLICATIONS

5.1. Research papers in referred journals

1. **Bobby Paul**., Rajesh M.K. and Naresh Kumar S. 2011. Isolation and characterization of a cDNA sequence encoding MAP kinase induced during water stress in coconut. (Accepted in Indian Journal of Horticulture).
2. **Bobby Paul**.,Rajesh, M.K., Kiran A.G., George T. and Naresh Kumar S. 2011. Differential expression of genes regulated in response to water stress in coconut. Full paper accepted in: Proceedings of the International Conference on Coconut Biodiversity for Prosperity. October 27-30, 2010. Central Plantation Crops Research institute, Kasaragod, Kerala.
3. **Bobby Paul,** Rajesh, M.K. and Naresh Kumar S. 2011. Bioinformatics approach towards mining of candidate genes for water stress in coconut. Full paper accepted in: Proceedings of the International Conference on Coconut Biodiversity for Prosperity. October 27-30, 2010. Central Plantation Crops Research institute, Kasaragod, Kerala.
4. **Bobby Paul**, Rajesh, M.K., Jayakrishnan, V.P., Ramesh Kumar, M.P. and George V Thomas. 2010. CMSD: A Database of Microsatellite Markers Genotyped in Indigenous Coconut Accessions. *Indian Journal of Plant Genetic Resources* **23**(1): 100-103.
5. Sreesmitha V., Rajesh M. K., **Bobby Paul**, Anitha Karun, Ramesh Kumar M. P., George V. Thomas. 2010. *In silico* prediction of function and modelling of WRKY protein in coconut. *Journal of Plantation Crops.* **39** (2): 144-147.

5.2. Papers presented in international/ national conference

1. **Bobby Paul,** M.K Rajesh, Kiran A.G, George Thomas and Naresh Kumar S. 2010. Differential expression of genes regulated in response to water stress in coconut. Accepted in: International conference on coconut biodiversity for prosperity. October 27-30, 2010. Central Plantation Crops Research institute, Kasaragod, Kerala.TS3-PO2: pp 53.
2. **Bobby Paul** andM.K Rajesh. 2010. Bioinformatics approach towards mining of candidate genes for water stress in coconut. In: International conference on coconut biodiversity for prosperity. October 27-30, 2010. Central Plantation Crops Research institute, Kerala. TS4-PO4: pp 71.
3. **Bobby Paul**, Rajesh M.K. and Naresh Kumar S. 2010. Isolation and characterization of a cDNA sequence encoding MAP kinase induce during water stress in coconut. In: Proceedings of Golden Jubilee National Symposium on Plant Diversity Utilization and Management, 27-29 May, 2009, Organized by Department of Botany, University of Kerala, Thiruvanathapuram (p.5.5-p.171).
4. **Bobby Paul**, Remya K, Rajesh M.K and Naresh Kumar S. 2010. Isolation, characterization and homology modeling of rbcL (ribulose-1, 5-bisphosphate carboxylase) large-subunit gene from *Cocos nucifera*. L. In: National consultative meet on Bioinformatics: An Agricultural perspective. 11-12 October, 2010. Indian Institute of Spices Research, Calicut. A-05 pp 106.
5. **Bobby Paul**, Rajesh M.K. and Naresh Kumar S. 2010. Diversity of rbcL gene sequences and its use in assessment of molecular phylogeny in Arecaceae. In: National Conference on Horticultural Bio-diversity for Livelihood, Economic Development and Health Care, 29-31 May, University of Horticultural Sciences, Bangalore (TA1:P-14-p.19).

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